# PHYSIOLOGY OF INSECT DIAPAUSE. X. AN ENDOCRINE MECHANISM FOR THE INFLUENCE OF TEMPERATURE ON THE DIAPAUSING PUPA OF THE CECROPIA SILKWORM

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Among the fifteen thousand Cecropia silkworms which we have reared during the past ten years, only an occasional individual has failed to begin a prolonged period of diapause immediately after pupation. Many of the animals underwent pupation in early summer, at the season when one might expect environmental temperature and photoperiod to favor uninterrupted growth and metamorphosis. Yet the intervention of diapause was no less uniform than in animals pupating in September or October. The Cecropia silkworm is therefore a typical single-brooded or "univoltine" insect.

The pupal diapause provides for the overwintering of the species. Though the pupa undergoes little or no morphological advance during this period, the months of exposure to the low temperatures of winter are not "time out." By its action within the diapausing insect, low temperature promotes certain physiological changes which assure the initiation of adult development the following spring. The detailed nature of these changes has been the central concern of the present study.

The investigation has been greatly assisted by a series of recent reviews surveying the voluminous literature pertaining to insect diapause (Andrewartha, 1952; Andrewartha and Birch, 1954; Lees, 1955). These reviews, to which the reader is referred for background to the present study, make clear that the potentiation of development by low temperature is a distinguishing feature of diapause, serving to differentiate diapause from hibernation, quiescence, and other forms of dormancy which are enforced by low temperature.

## Materials and Methods

The investigation was performed on pupae of the Cecropia silkworm (*Platysamia cecropia* L.). The larval stages were reared under large nylon nets on wild-cherry trees. The cocoons were harvested immediately after spinning and placed at 20 to 25° C. for at least six weeks prior to use. Pupation occurred during the

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first two weeks and was followed by the characteristic decline in metabolic rate to the extremely low diapausing level (Schneiderman and Williams, 1953).

The temperature relations of several other species of diapausing saturniids have also been studied in detail. However, in the present report attention centers on the Cecropia silkworm, and only one series of experiments will be described on the related species, *Samia walkeri* (*cynthia*).

Ten years ago, at the outset of the present study, it was already evident that a quantitative approach to the problem required the use of a rather large number of individuals in testing each condition and circumstance. This practice, in turn, complicated the management of the experimental findings, since the latter sometimes showed considerable scatter. The procedure which was finally adopted and applied throughout the present study was to define the mean of each distribution, as well as the latter's standard deviation, by the use of probability paper (Codex

TABLE I

*Previously unchilled Cecropia pupae at each of a series of constant temperatures: time for initiation of development and for adult development itself*

| Temp. | No. animals | Weeks to initiate development | | Weeks to complete development | | Total Weeks |
|---|---|---|---|---|---|---|
| | | Mean ± S.D. | Recip. × 100 | Mean ± S.D. | Recip. × 100 | |
| 2½° C. | 20 | ∝ | 0 | ∝ | 0 | ∝ |
| 6 | 20 | ca. 41 | 2.6 | ∝ | 0 | ∝ |
| 10 | 18 | 25.0 ± 1.9 | 4.0 | 16.3 ± 2.0* | 6.1 | 41.3 |
| 15 | 19 | 23.3 ± 3.9 | 4.3 | 11.9 ± 1.4 | 8.4 | 35.2 |
| 20 | 20 | 37.0 ± 9.4 | 2.7 | 4.9 ± 0.6 | 20.4 | 41.9 |
| 25 | 20 | 25.4 ± 4.9 | 3.9 | 3.1 ± 0.9 | 32.3 | 28.5 |

* Adult moths with extremely dark pigment.

Book Co., Norwood, Massachusetts, No. 32,451, Normal Ruling). The application of this technique, as well as various other experimental procedures, will be described in the sections that follow.

## RESULTS

### 1. *Unchilled pupae at specific constant temperatures*

#### A. Initiation of adult development

When unchilled Cecropia pupae are placed at a particular temperature within the range 6 to 35° C. and maintained thereafter at this same constant temperature, the pupal diapause is ultimately terminated and adult development begins. In no case, however, does this occur immediately. Irrespective of what the temperature may be, adult development, as signalled by the retraction of the epidermis from the overlying pupal cuticle, is initiated only after prolonged exposure to the constant temperature. Prior to the actual initiation of adult development, even a detailed dissection of the pupa fails to reveal any clear-cut change from its condition at the outset.

In order to appraise the matter in quantitative terms, a study was made of the

time required for the initiation of adult development at each of a series of constant temperatures. For this purpose, a homogeneous batch of animals (which had pupated 6 to 8 weeks earlier and has subsequently been stored at 20–25° C.) was removed from cocoons, placed in individual capped "creamers," and distributed among seven temperature cabinets or constant temperature rooms at temperatures ranging from 2½ to 30° C. Thereafter at weekly intervals, each pupa was checked for the initiation of adult development, as judged by the retraction of the leg epidermis.

In the experimental results summarized in Table I, it will be observed that a considerable period was required for the initiation of adult development at all of

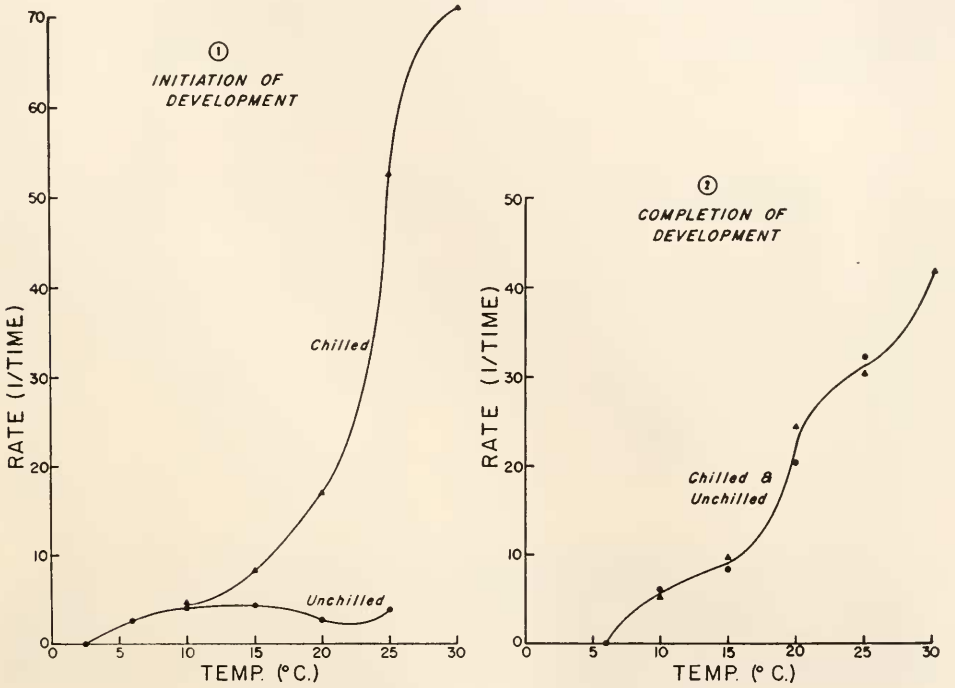


FIGURE 1. The rate (100/Weeks) of initiation of adult development at each of a series of constant temperatures. The lower curve describes the response of previously unchilled pupae; the upper curve, the response of pupae which had been chilled for 15 weeks at 6° C. prior to the experiment. The period of preliminary chilling greatly accelerates the initiation of development.

FIGURE 2. The rate (100/Weeks) of adult development at a series of constant temperatures after development has begun. The response of previously unchilled pupae (circles) cannot be distinguished from that of previously chilled pupae (triangles).

the constant temperatures within the range 2½ to 30° C. Indeed, at 2½° C. adult development never began, and pupae at this temperature invariably died after about a year.

In the lower curve in Figure 1 the results have been computed and plotted on a rate (1/Time) basis. The relatively minor effects of temperature are here clearly evident. Even these minor effects are paradoxical in that the rates encountered at 20° and 25° C. are actually lower than those at 10° and 15° C.

B. Completion of adult development

Each animal in the experiment just considered was permitted to continue to develop and to emerge as an adult moth. In this manner it was possible to ascertain the effects of temperature on the kinetics of adult development itself. The results are recorded in Table I, and plotted on a rate (1/Time) basis in Figure 2.

It is clear that once adult development has begun, a striking change occurs in the insect's response to environmental temperature. Temperature now becomes a prime determinant of the rate of adult development—a fact which is signalled in Figure 2 by the curve's pronounced and positive slope.

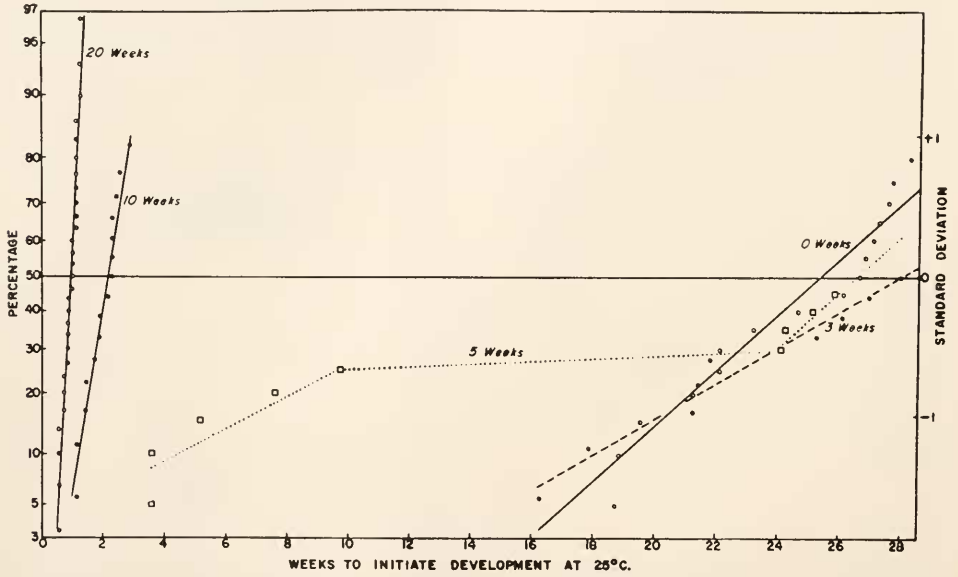


FIGURE 3. Time required for the initiation of adult development at 25° C. after preliminary exposure to 6° C. for 0–20 weeks. By the use of a probit scale on the Y-axis, each distribution is converted into a straight line. The discontinuous line for the 5-week series signals a bimodal distribution.

Though the formation of the adult moth, as noted in the preceding section, ultimately begins after about ten months at 6° C., such animals never proceed beyond early development and ultimately die before reaching a stage corresponding to that which would be attained at 25° C. after only three days. At the slightly higher temperature of 10° C., approximately 3½ months elapse between the initiation and completion of development. Adult moths developing at this constant temperature are extremely dark, the reddish pigments of scales and hairs being replaced by black and the white pigments by gray. At 10° C. the insect is incapable of sufficient muscular effort to emerge spontaneously from the pupal exuviae and ordinarily does not do so unless returned to higher temperatures. By contrast, at 15° C. and all higher temperatures up to 30° C., the moths show normal pigmentation and are able to emerge spontaneously and spread their wings. At the still higher temperature of 35° C., development proceeds with considerable rapidity, but

the moths are extremely pale and feeble, and female individuals show few if any eggs.

*2. Previously chilled pupae at specific constant temperatures*

The experiment described in the preceding section was repeated on a homogeneous batch of pupae which had been previously exposed to 6° C. for 15 weeks. The results are summarized in Table II and plotted on a rate (1/Time) basis in Figures 1 and 2.

The upper curve in Figure 1 makes clear the extraordinary difference in the temperature response of chilled pupae from that previously defined for unchilled pupae (lower curve). Temperatures above 10° C. now greatly accelerate the initiation of adult development. For example, at 25° C. the time required for the initiation of adult development is decreased from 25 weeks to 13 days.

TABLE II

*Previously chilled pupae (6° C. for 15 weeks) at each of a series of constant temperatures: time for initiation of development and for adult development itself*

| Temp. | No. animals | Weeks to initiate development* | | Weeks to complete development | | Total Weeks |
|---|---|---|---|---|---|---|
| | | Mean ± S.D. | Recip. × 100 | Mean ± S.D. | Recip. × 100 | |
| 10° C. | 8 | 21.4 ± 7.4 | 4.7 | 19.1 ± 4.3 | 5.2 | 40.5 |
| 15 | 19 | 12.1 ± 14.1 | 8.3 | 10.3 ± 0.9 | 9.7 | 22.4 |
| 20 | 19 | 5.9 ± 4.7 | 17.0 | 4.1 ± 1.9 | 24.4 | 10.0 |
| 25 | 30 | 1.9 ± 1.0 | 52.7 | 3.3 ± 0.3 | 30.3 | 5.2 |
| 30 | 19 | 1.4 ± 1.0 | 71.4 | 2.4 ± 0.3 | 41.7 | 3.8 |

* Not counting the previous 15 weeks at 6° C.

Attention is directed to the tempo of adult development after the latter has begun (Fig. 2). Manifestly, the curve descriptive of the response of previously chilled pupae cannot be distinguished from that recorded for unchilled pupae. We are therefore led to infer that the pupal diapause, as a physiological and biochemical phenomenon, is primarily concerned with events which culminate in the initiation of development, and that it is little concerned with adult development itself. Our present problem, in brief, is to account for the differences between the upper and lower curves in Figure 1.

One can say immediately that the upper curve, which describes the response of previously chilled pupae, is not remarkable: it is a familiar experience to find biological processes accelerated by temperature. The previously chilled pupa, in effect, is responding to temperature like a normal non-diapausing insect. But what is remarkable is the effective "insulation" of the previously unchilled pupa from the accelerating action of temperature. An explanation of this effect was the primary objective of the present study.

*3. Graded exposure of unchilled pupae to 6° C.*

We have already considered and illustrated in Figure 1 the way in which preliminary exposure to 6° C. greatly decreases the time required for the initiation of

development at 25° C. The speed with which the response ensues at 25° C. becomes a measure of the persistent effects of the previous exposure to low temperature. In the experiment recorded in Figure 1, the exposure to 6° C. was for 15 weeks. However, the same technique obviously can be used to appraise the quantitative effects of any other duration of chilling. This maneuver has been used extensively during the present study in order to probe the latent effects of low temperature.

Figure 3 summarizes a part of the results of a detailed study of this type in which the effects of the duration of exposure to 6° C. were studied. The experiment was performed on 160 Cecropia pupae which were harvested in freshly spun cocoons on August 7–11, 1953, and temporarily stored at 25° C. for ten weeks prior to the experiment. The cocoons were then placed in capped cardboard containers and stored at 6° C. Thereafter, at regular intervals ranging from 0 to 45 weeks a group of twenty pupae were removed from cocoons, placed in individual, uncapped "creamers," and returned to 25° C. The time which each animal required to initiate adult development was then ascertained in terms of the initiation of retraction of the leg epithelium—a signal of the second day of adult development at 25° C.

The effects of the graded exposures to 6° C. are plotted in Figure 3 on a probability grid, whose use was mentioned in the section on Methods. This type of plot has the advantage of transforming normal distributions into straight lines; it likewise permits one to define the mean and the standard deviation with far greater precision than is possible by any simple arithmetic procedure. In Figure 3 a straight line has been drawn by inspection through the distribution of responses after each preliminary exposure to 6° C. It is immediately apparent that 3 weeks at 6° C. failed to facilitate the subsequent response at 25° C.; indeed, the mean (28 weeks) was slightly prolonged beyond the value after no chilling (25.4 weeks); moreover, as signalled by the flatter slope to the curve, the scatter in the data (which was already large after no chilling) was further enhanced after three weeks at 6° C.

In Figure 3 attention is now directed to the distribution of responses at 25° C. after preliminary exposure to 6° C. for 10 and 20 weeks, respectively. It is clear that all these data fall nicely into straight lines whose steep slopes signify minor degrees of scatter. It is of particular interest and importance to note that the means, thus defined, are extremely small and range from 7 to 15 days. Consequently, a spectacular effect of the low temperature treatment has taken place between the third and tenth weeks at 6° C.

Within this critical period the response was examined after 5 weeks of chilling. In the results recorded in Figure 3, it is necessary to place a discontinuous line through the data thus obtained. This signifies that the distribution of responses is bimodal. Five of the twenty pupae, irrespective of sex, showed a significant effect of the low temperature treatment. An extrapolation to the 50 per cent level of a line joining these five points suggests a mean value of approximately 16 weeks for the initiation of development. But for the next five animals to initiate development, it is clear that 5 weeks at 6° C. was without detectable effect, the estimated mean being, in fact, slightly greater than that observed after no chilling.

The response after 5 weeks of chilling is therefore of particular interest in that 25 per cent of the pupae showed a significant influence of the preliminary low

temperature treatment while 75 per cent did not. Since the response after 10 weeks of chilling is homogeneous, it follows that in the normal course of events, this residual 75 per cent must shift from the delayed to the accelerated type of response between the fifth and tenth weeks at 6° C.

A point of considerable theoretical interest is the lack of overlap between any regions of the 5-week and 10-week curves in Figure 3. This signifies that even in the five animals which responded most promptly in the 5-week series, the physio-

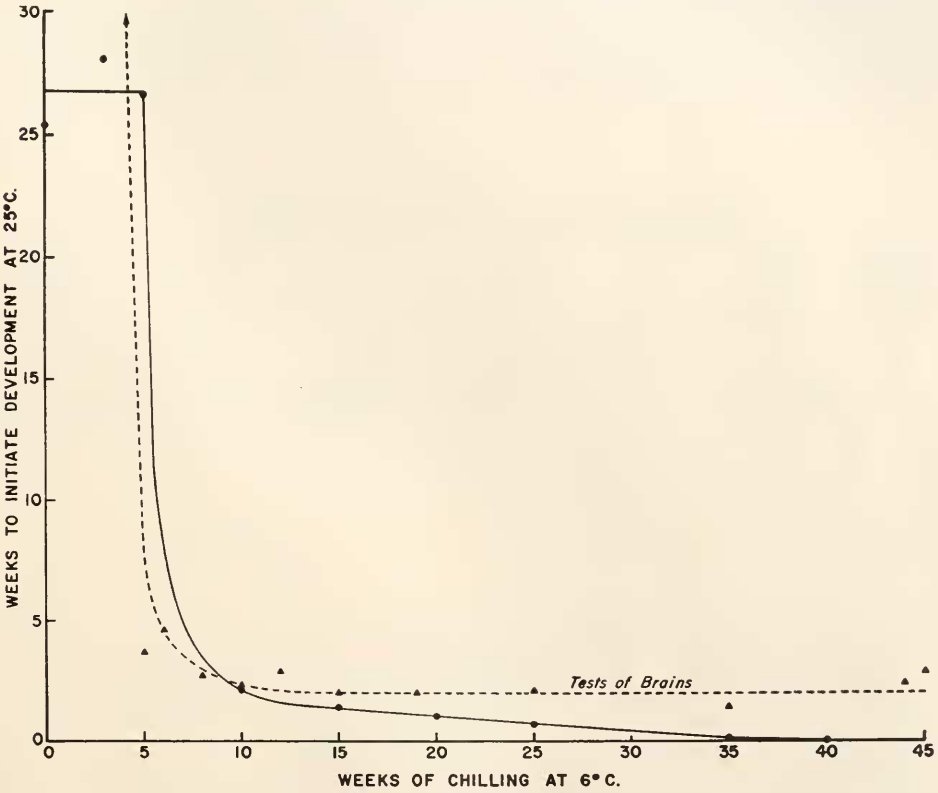


FIGURE 4. The unbroken curve records the time required for the initiation of development at 25° C. as a function of the duration of preliminary exposure to 6° C. The broken line records the corresponding changes occurring in the endocrine activity of the pupal brain. For explanation, see text.

logical changes responsible for the accelerated response had not proceeded to completion by the fifth week, but, so to say, were caught "in transit."

On the basis of the results just considered, one can therefore recognize a sequence of two relatively stable states during pupal life, corresponding, respectively, to the *delayed* and the *accelerated* responses to the warm temperature. Moreover, the transition from the first to the second state shows the kinetics of a threshold reaction, being initiated by each individual during an apparently brief period after the third week at 6° C. and prior to the tenth week. It is equally clear that this

reaction, which then effects the transition, can scarcely be "all or none," since it requires at least several weeks to pursue its full course.

These various conclusions are illustrated in Figure 4 where the mean times required for the initiation of development at 25° C. have been read from Figure 3 and plotted as a function of the duration of preliminary chilling at 6° C. The abrupt descending limb of the curve has been placed just after the fifth week at 6° C., though, for reasons mentioned above, its true position for about one-fourth of individuals is slightly to the left of this point.

Notwithstanding persistent uncertainties with respect to the precise time-course of the descending segment of the curve, there can be little doubt that the relationship is dominated by the transition of the pupa from a state of delayed response at 25° C. to one of accelerated response. This transition, we have inferred, is com-

TABLE III

*Time* for initiation of development of Cecropia pupae at 25° C. after indicated exposures to specific constant temperatures*

| Exposure (weeks) | 2½° C. (weeks) | 6° C. (weeks) | 10° C. (weeks) | 15° C. (weeks) | 20° C. (weeks) | 30° C. (weeks) |
|---|---|---|---|---|---|---|
| 0 | 25.4 ± 4.9 | 25.4 ± 4.9 | 25.4 ± 4.9 | 25.4 ± 4.9 | 25.4 ± 4.9 | 25.4 ± 4.9 |
| 3 | — | 28.1 ± 7.6 | 24.6 ± 4.7 | — | — | — |
| 5 | 28.0 ± 10.6 | 26.7** | 20.9** | 16.0** | 23.7 ± 12.9 | 22.6 ± 2.7 |
| 10 | 26.3 ± 15.0 | 2.1 ± 0.7 | 1.7 ± 0.3 | 2.0 ± 0.4 | 5.3** | 25.0 ± 20.0 |
| 15 | 2.1** | 1.4 ± 0.7 | 1.0 ± 0.4 | 1.1 ± 0.6 | 3.4 ± 1.9 | 26.0 ± 19.1 |
| 20 | — | 1.0 ± 0.3 | — | — | — | — |
| 23 | — | — | — | Zero*** | — | — |
| 25 | 0.6 ± 0.3 | 0.7 ± 0.3 | Zero*** | — | — | — |
| 35 | — | 0.1 ± 0.1 | — | — | — | — |
| 37 | — | — | — | — | Zero*** | — |
| 40 | — | Zero*** | — | — | — | — |

* Each datum is the mean ± standard deviation (in weeks) of 18–20 pupae, and was derived from a plot of the individual responses on probit paper.

** Bimodal distributions. The mean characteristic of the majority of the sample is recorded.

*** Development is initiated under conditions of constant exposure to the temperatures noted.

plete by the tenth week at 6° C. Yet it will be observed that after the tenth week of chilling a slow downward trend continues in the lower horizontal limb of the curve in Figure 4. Thus, after 35 weeks at the low temperature, adult development begins after only one day at 25° C. And after approximately 40 weeks, adult development actually begins at 6° C. It is equally clear, however, that the changes after the tenth week are extremely slow compared to those occurring during the first ten weeks at 6° C. Moreover, there are substantial reasons, to be considered below, for separating the early and the late effects and attributing the gentle slope of the lower horizontal part of the curve to secondary changes within the fully "accelerated" pupa.

4. *Potentiation of development by temperatures other than 6° C.*

We have considered up to this point the potentiation of development by prior exposure to 6° C. The same technique permits one to examine the latent effects

of any other temperature within the physiological range. The temperatures selected for study in this manner were 2½, 10, 15, 20 and 30° C., the effects in each case being judged in terms of the time subsequently required for the initiation of adult development at 25° C. In order to facilitate comparisons with the results at 6° C., unchilled diapausing pupae of the same 1953-brood were utilized and all experiments were set up synchronously. The over-all results, including those previously described for 6° C., have been summarized in Table III.

In Figure 5 the data have been computed on a rate (1/Time) basis and plotted as a series of asymmetric, bell-shaped curves. Each curve records the rate with

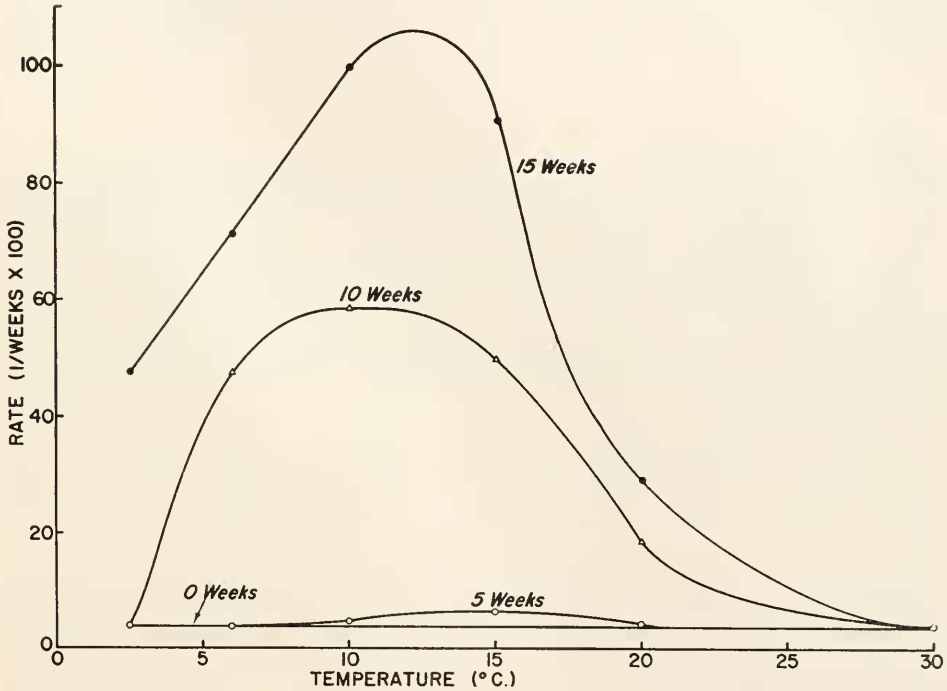


FIGURE 5. The rate (100/Weeks) of initiation of adult development at 25° C. after preliminary exposure to 2½–30° C. for 0–15 weeks.

which adult development is initiated at 25° C. after a particular period of preliminary exposure to temperatures ranging from 2½ to 30° C. For comparison, the corresponding rate in the absence of any preliminary treatment has been recorded as the straight line paralleling the X-axis.

Exposure to any of these temperatures for less than 5 weeks is without detectable effects on the subsequent rate of development at 25° C.—a conclusion already noted in the experiments at 6° C. After 5 weeks of the preliminary treatment, the distribution of results becomes bimodal at 6, 10, and 15° C. (see Table III). In Figure 5 this fact is not evident in the 5-week curve because the plotted points describe the primary mode in each of the three bimodal distri] ations. However, the

bimodality is of interest in itself because it signals the onset of a period of rapid change.

This inference is confirmed in the results recorded after 10 weeks of preliminary treatment. Here a pronounced potentiation of development is apparent after exposure to 6–15° C. And, here also, the results at 20° C. become bimodal (Table III). A continuation of this trend is evident after 15 weeks of exposure, when, for the first time, the range of effective temperatures is broadened to include 2½° C. And, finally, as recorded in Table III, adult development actually begins by the 25th week of continuous exposure to 10–15° C.

The results as a whole are therefore in general agreement with the analysis suggested in connection with the experiments at 6° C. It is of particular interest that an intermediate range of temperatures (6 to 15° C.) is far more effective in potentiating development than are temperatures lower than 6° C. or higher than 15° C.

5. *Effects of brain removal*

The analysis, up to this point, has focussed attention on a physiological change within the diapausing insect which mediates the catalytic effects of low temperature. As long as ten years ago there were already substantial reasons for localizing the site of this physiological change in the pupal brain. It was found that low temperature alters the pupal brain in such a manner that the brain regains its competency to secrete a hormone prerequisite for the initiation of adult development (Williams, 1946). It was also possible to show that the secretion of this hormone is necessary until adult development actually begins. Consequently, if one removes the pupal brain prior to the initiation of development the pupa is stabilized in a state of "permanent diapause."

Manifestly, these previous findings have an intimate bearing on the results of the present study. If the action of low temperature is mediated solely by the brain, then it should be possible to "excise" the effects of prior chilling by removing the brain.

Experiments of this type have been performed on a considerable scale during the past ten years. The results can be summarized most briefly in the statement that among several thousand chilled pupae from which the brains were removed prior to the initiation of development, not a single individual escaped from diapause and initiated development.

Consider, for example, pupae chilled for 35 weeks at 6° C. Though such animals, as illustrated in Table III, initiate development after only one day at 25° C., development is permanently blocked if the brain is removed during those first 24 hours. Such brainless animals continue to live at 25° C. for up to two years thereafter, and during this period cannot be distinguished morphologically from brainless unchilled pupae. By this and analogous procedures it was possible to confirm the fact that the effects of prior chilling can be eliminated by excision of the brain.

Whereas it was initially thought that the brain is no longer necessary after the earliest indication of adult development (Williams, 1946), more detailed studies showed that the brain continues to be required during the first 24 hours of adult development, and, indeed in rare individuals, during the first 48 hours (Williams, 1952). It is of particular interest that excision of the brain during the first 24 hours blocks the further progress of development. Dissection of such animals

showed that the epidermis (which had already loosened its attachments to the pupal cuticle and begun to retract) re-appressed itself to the cuticle and re-established its extremely intimate connections. In effect, therefore, the insect over a period of several weeks returns to a state indistinguishable by any morphological sign from that of a diapausing pupa.

An occasional animal from which the brain had been removed during the second day of development showed an anomalous response in that development of certain specific regions, including head, thorax, wings, legs, and antennae, continued for a time and then stopped with these parts in a more or less advanced stage of adult differentiation. But in virtually all cases where brain removal was postponed until after the first 48 hours of adult development, the latter continued to yield brainless but otherwise normal moths.

6. *Quantitative changes in brain activity during chilling*

The experiments just considered point to the pupal brain as the vehicle by which low temperature catalyzes the termination of diapause. Under this circumstance one would anticipate systematic changes in the brain's endocrine activity to account for the quantitative aspects of the low temperature effect; *i.e.,* for such changes in development potential as one observes in Figures 4 and 5.

The most detailed study of this sort was performed in 1953–1954 in synchrony with the above-mentioned temperature studies and on aliquot samples of the same homogeneous group of animals. At the outset of the experiment the brains were removed from ten unchilled individuals and tested for activity by implanting them under a plastic window at the tip of the abdomen of a corresponding number of brainless unchilled pupae. Subsequently, at intervals of one to several weeks, the brains of additional 6° C.—animals were removed and tested in like manner. By this procedure it was possible to quantitate the endocrine activity of each implant in terms of the time which it required to evoke the development of the host at 25° C.

The results are summarized in Table IV. It is evident that only an occasional brain was active when obtained from pupae chilled for less than 5 weeks; moreover, in such instances, development ordinarily began only after a considerable delay (up to 40 weeks). By contrast, brains chilled for 5 weeks or longer were not only active in a high proportion of cases, but also, with rare exception, evoked the initiation of adult development within a month after implantation. Evidently, on or about the eighth week at 6° C., the brain attains maximal activity, as judged by the high percentage and uniform rapidity of the responses. Presumably, the occasional inactivity encountered after the eighth week was caused by injury to the brain during the latter's excision and transplantation.

It is particularly fruitful to compare the change in the brain's activity with the previously described behavior of intact pupae after systematic exposure to 6° C. To this end, the mean values in Table IV have been plotted as the broken-line curve in Figure 4. The degree of congruity between these curves leaves little doubt that the abrupt shift in the developmental potential of the pupa as a whole is the outcome of an equally abrupt shift in the endocrine activity of the brain.

Notwithstanding the substantial correlation between the two curves in Figure 4, one also observes minor but highly significant differences. Thus, after chilling for less than 5 weeks, only about one brain in three was found active when tested by

implantation. Yet, even in the absence of chilling, the undisturbed brains of normal pupae exert their effects during the first year at 25° C. This is one of the few differences encountered between the endocrine activity of "loose" implanted brains and brains retaining normal nerve connections. However, such connections are clearly not obligatory since briefly chilled implants were able to achieve threshold activity in one-third of the cases.

A second difference between the curves in Figure 4 is the fact that 50 to 70 per cent of excised brains showed full activity when tested after 5 or 6 weeks of chilling, whereas only about 25 per cent of unoperated pupae developed promptly after the same amount of chilling. Since this period coincides with the time of

TABLE IV

*Tests* of endocrine activity of brains from pupae previously chilled at 6° C. for specific periods*

| Prior chilling of brains (weeks) | Number tested | Number active | Time** to evoke development at 25° C. (weeks) |
|---|---|---|---|
| 0 | 10 | 2 | 10; 11; – — |
| 3 | 10 | 4 | 3.3; 3.3; 23.9; 40.6; — |
| 4 | 10 | 3 | 2.7; 3.0; 37.9; — |
| 5 | 20 | 14 | 3.7 ± 1.1 |
| 6 | 10 | 5 | 4.6 ± 1.9 |
| 8 | 10 | 7 | 2.7 ± 1.3 |
| 10 | 10 | 10 | 2.3 ± 0.6 |
| 12 | 10 | 7 | 2.9 ± 1.1 |
| 15 | 10 | 10 | 2.0 ± 0.9 |
| 19 | 16 | 16 | 2.0 ± 0.7 |
| 25 | 8 | 8 | 2.1 ± 0.6 |
| 35 | 10 | 9 | 1.4 ± 0.3 |
| 44 | 9 | 8 | 2.4 ± 1.7 |
| 45 | 22 | 19 | 2.9 ± 1.1 |

* Each brain was implanted into a brainless diapausing pupa and the time for the latter to initiate adult development at 25° C. determined.

** Mean ± standard deviation in weeks are recorded for all groups in which at least 50 per cent of animals developed.

rapid change in brain activity, conceivably the difference is a sampling artifact attributable to the limited number of animals tested. However, it is even more likely that the difference is real and that, by some unknown means, the surgical manipulation of the brain during this critical period hastens to completion the changes which restore the brain's endocrine activity.

A final difference between the two curves in Figure 4 concerns the lower segments after the tenth week of chilling. It is clear that after 8–10 weeks at 6° C. the brain has fully regained its endocrine powers and that only minor changes occur thereafter. By contrast, in normal unoperated pupae the onset of development at 25° C. is progressively accelerated when the low temperature treatment is prolonged beyond the tenth week; indeed, we have already observed that adult development actually begins at 6° C. after about 40 weeks. This implies that a slow release of the brain hormone takes place at the low temperature and that a threshold titer

is ultimately built up after about 40 weeks at 6° C. Consequently, after 35 weeks at 6° C. the titer is barely sub-threshold and further brain function at 25° C. is required for only one day. Since the excised brains were tested in brainless unchilled pupae lacking any substantial titer of brain hormone, one would anticipate the observed difference between the lower segments of the curves in Figure 4.

On the basis of these various observations, the effects of low temperature on the intact diapausing insect are subject to interpretation. Low temperature promotes a transformation of the brain so that the latter becomes competent to secrete its hormone; as illustrated in Figure 4, this process is complete by the tenth week at 6° C. The slow secretion of the hormone then begins at the low temperature. Once the brain has regained its endocrine powers, then, as illustrated in Figure 1, the secretion of the hormone is actually retarded by low temperature. The catalytic effects of low temperature on the diapausing pupa may therefore be said to end as soon as low temperature has induced the physiological transformation of the brain.

*7. In vivo and in vitro chilling of the brain*

In the course of the routine preparation of several thousand brainless diapausing pupae used in the studies of the present series, the excised unchilled brains were often implanted into brainless diapausing hosts and then subjected to chilling under conditions of *in vivo* culture. In this manner it was possible to build up a "brain bank" which was useful for various purposes. Such brains, after a suitable period of chilling, could then be reclaimed and utilized as active chilled brains.

This finding, in itself, gives assurance that the normal connections of the brain with the rest of the nervous system are not prerequisite for the temperature effect. However, as Andrewartha (1952) has pointed out, the experimental method did not exclude the possibility that low temperature acts indirectly on the brain—perhaps by making available within the host some precursor of the brain hormone.

Under this latter circumstance one would expect the hypothetical activating substance to be present in high concentration in pupae chilled for a prolonged period. This prospect was tested by excising the brains of previously chilled pupae and reimplanting one or more brains of unchilled pupae. In no case did the previously chilled host have any detectable effect in activating the unchilled implant. Manifestly, this result argues against the view that low temperature acts by promoting the release of some brain-stimulating factor within the pupa as a whole. It also opposes an alternative possibility that low temperature destroys a brain-inhibitor within the pupa as a whole.

A conclusive test of the direct action of low temperature on the brain obviously requires that the brain be activated apart from the rest of the insect; *i.e.*, under conditions of *in vitro* culture. This objective is not easy to realize in view of the unsatisfactory state of knowledge concerning the culture of insect tissues other than blood cells and spermatocytes (Schmidt and Williams, 1953).

Nevertheless, during the past ten years a considerable number of unchilled brains have been subjected to *in vitro* culture under sterile conditions, the media consisting of various types of physiological solutions or of blood obtained from chilled, unchilled, or developing animals. The blood-containing media invariably underwent darkening during prolonged exposure to low temperature, notwithstanding the presence of crystals of the potent anti-tyrosinase, phenylthiourea. Darkened

blood is known to be extremely toxic to insect tissues, and such experiments were doomed to failure.

In no case did the brains survive the low temperature culture for longer than about two weeks. Such brains, when implanted into brainless pupae, were immediately attacked by phagocytic cells and ultimately broken down.

One might suppose that even dead brains, if sufficiently numerous, might contribute a critical concentration of hormone after implantation. This possibility was discounted by the finding that active brains, killed by freezing and thawing in liquid nitrogen or by exposure to temperatures higher than 50° C., were ineffective when implanted in large numbers. Evidently, only a trace of hormone is stored in the normal active brain, since the biological effect of a single hemisphere of a living brain cannot be duplicated by the implantation of fifty dead brains.

Consequently, for reasons that are believed to be technical in character, it has been impossible to accomplish this final and most direct test of the view that low temperature achieves its catalytic effect by its direct action on the brain. However, the sum total of available evidence gives little reason to think otherwise.

8. *Winter sickness*

It will be recalled that the onset of adult development under conditions of constant exposure to 6° C. is delayed until approximately the fortieth week at the low temperature. Even then, the development which takes place fails to proceed beyond an early stage, corresponding to that attained after only two days of adult development at 25° C. If the exposure to 6° C. is continued still longer, one detects the onset of a curious syndrome for which we suggest the name "winter sickness." The distinctive feature is a progressive paralysis of the intersegmental muscles of the abdomen; *i.e.,* the muscles responsible for the "tone" of the motile abdominal segments and the latters' characteristic circular motion in response to mechanical stimulation.

In pupae returned to room temperature after 46 weeks at 6° C., the tone of these muscles is subnormal for several days thereafter, and contraction can be evoked only when the pupa is subjected to mechanical stimulation such as squeezing. Further prolongation of the low temperature treatment leads to a flaccid paralysis of all muscles save the heart. As summarized in Table V, electrical excitability likewise disappears, and contraction can no longer be induced in response to tetanizing shocks administered to the abdomen by overlying electrodes.

The early stages of winter sickness are reversible at room temperature until about the fifteenth month at 6° C. (see Table V). After several days at 25° C., electrical excitability returns, followed shortly therafter by motion in response to mechanical stimulation, and, finally, by the recovery of tone. Adult development then continues where it had left off. However, if the exposure to 6° C. has been for longer than a year, the adult moths, when formed, are generally feeble and unable to spread their wings, or, indeed, to escape from the pupal exuviae. And in the case of female individuals only a few eggs are matured.

After 15 months at 6° C., winter sickness becomes irreversible for a progressively larger number of individuals. Such animals show the absence of heart beat when returned to room temperature, and, in fact, are already dead, as judged by the lack of any detectable oxygen consumption.

Winter sickness is not peculiar to the Cecropia silkworm, since it has been regularly encountered in all species of diapausing pupae which we have studied at low temperatures. For example, *Telea polyphemus* shows reversible sickness after 60 weeks at 6° C. and death after 90 weeks; *Samia walkeri* (*cynthia*) shows flaccidity after 35 weeks at 6° C. and death after 90 weeks. *Antheraea mylitta* and *Actias selene* are even more susceptible, being killed by 6 months at 6° C.

A considerable number of experiments were performed in search of the physiological basis of winter sickness. Of particular interest was the paralysis of the abdominal muscles—a condition which one fails to duplicate even by the injection of such potent agents as curare or botulinus toxin.

TABLE V

*Winter sickness after prolonged exposure to 6° C.*
(*Platysamia cecropia*)

| Weeks at 6° C. | No. pupae | Stage of adult development after chilling | Behavior at 25° C. |
|---|---|---|---|
| 42 | 40 | 0–1 day | Lively; prompt development |
| 44 | 20 | 0–1 | Lively; prompt development |
| 46 | 25 | 0–1 | Induced motion only; prompt recovery and development |
| 49 | 40 | 0–1 | 30% show flaccid paralysis and electrical inexcitability at outset; all recovered and developed |
| 52 | 50 | 0–1 | 50% flaccid and inexcitable at outset; all recovered and developed |
| 57 | 90 | 0–1 | 70% flaccid and inexcitable at outset; all recovered and developed into feeble moths |
| 66 | 40 | 0–2 | All flaccid and inexcitable at outset; only 60% recovered and developed into feeble moths |
| 70 | 65 | 0–2 | All flaccid and inexcitable at outset; only 50% recovered and developed into feeble moths |
| 74 | 10 | 0–2 | All flaccid and inexcitable at outset; only 25% recovered and developed into feeble moths |
| 90 | 50 | 0–2 | All flaccid and inexcitable; none recovered |

It was first thought that winter sickness might be peculiar to animals showing early adult development. This prospect was negated by the finding that at 2½° C., in the absence of any trace of adult development, the onset of winter sickness is even more prompt than at 6° C. This same effect was noted in other species. Indeed, in *Samia walkeri* (*cynthia*) winter sickness becomes irreversible after only 7 months at 2½° C.

A clue as to the physiological basis of winter sickness was provided by the following experiment performed on *Samia walkeri* (*cynthia*). From seven individuals showing reversible winter sickness the blood was drained and exchanged with that of a corresponding number of normal diapausing pupae. All animals were then maintained at 25° C. Within 12 hours the normal pupae receiving the winter-sick blood showed full and complete paralysis of the abdominal muscles. Electrical excitability also disappeared and reappeared only 3–5 days later. Recovery was synchronized with the formation of a chalky precipitate, which settled

on the plastic window at the tip of the abdomen and also deposited itself in the lumen of wings, antennae, and the body cavity as a whole.

The winter-sick pupae which received normal blood likewise recovered in 3–5 days, though whether this was hastened by the transfusion of normal blood cannot be stated in the absence of suitable controls. No precipitate formed in this blood and none was detected in the adult moths that ultimately formed.

In effect, therefore, it was possible passively to transfer the winter sickness by the transfusion of winter-sick blood. Moreover, the detoxification of this blood was accompanied by the formation of a copious white precipitate, presumably containing ureates and other insoluble end-products of nitrogenous metabolism.

*Samia walkeri* (*cynthia*) is a species which never escapes from diapause if maintained at temperatures higher than 20° C. After 15 months at 25° C., the normal, lively pupa shows the formation and deposit of the same sort of precipitate as mentioned above. Evidently, the intermediate metabolism of the pupa at the warm temperature is able to cope with nitrogenous end-products by detoxification and "excretion by precipitation," whereas, at temperatures below 10° C., the production of soluble end-products outbalances their detoxification. Winter sickness, in this sense, appears to be a genuine case of auto-intoxication within the "closed system" of the pupal insect, and to bear a resemblance to the mammalian syndrome of uremia. The phenomenon obviously merits further study.

## Discussion

On the basis of the experimental results set forth in the preceding sections, it is clear that the catalytic effects of low temperature on the diapausing insect are mediated by an endocrine mechanism. This mechanism centers in the brain. Previous studies had already demonstrated that the brain loses its endocrine powers just prior to the pupal moult and is inactive in the freshly pupated insect (Williams, 1946, 1952). We now see that the rate with which the brain recovers its activity is largely determined by environmental temperature.

By an apparently direct action on the brain itself, temperatures within the range 6 to 15° C. greatly accelerate the functional recovery of the brain. As soon as the brain has undergone this physiological change, the catalytic effects of low temperature are at an end. The pupa, equipped with an endocrinologically competent brain, becomes, in effect, the equivalent of a non-diapausing pupa.

It is fruitful to consider the similarities between two such animals; *i.e.*, between chilled diapausing pupae and unchilled non-diapausing pupae. Thus, in both types of animals the actual secretion of the brain hormone, as well as the development that follows, are favored by high temperature and retarded by low temperatures. Moreover, in both types of animals one can ordinarily establish or re-establish a pupal diapause by prompt excision of the pupal brain (Williams, 1952). And, finally, in both types of pupae, low temperature is still capable of enforcing a developmental standstill which is reversible at higher temperatures. On the basis of this comparison it seems necessary to conclude that the end-point of the pupal diapause is physiological in character, and is signalled by the functional recovery of the brain.

If attention is now directed to the overwintering of the diapausing insect, it is clear that exposure to temperatures within the optimal range of 6 to 15° C. ordinarily continues beyond the 10 weeks required for the full and complete recovery

of the brain. Hence, under normal circumstances, the dormant state begins as a "diapause" and continues thereafter as a "quiescence" (Andrewartha, 1952; Lees, 1955). The net effect is that the overwintering insect is poised to react to the warm temperatures of spring.

From the physiological point of view, attention focusses on the brain as the vehicle of the low temperature effect. Indeed, within the substance of the pupal brain, it is reasonable to pin-point the temperature effect even more closely within the 26 neurosecretory cells which secrete the brain hormone. Low temperature apparently accelerates the functional recovery of these cells from their inactive state in the diapausing pupa.

The activating reaction, itself, pursues a typical time-course as dictated by temperature. Critical exposure to low temperature is first required, following which the activation proceeds to completion within a period of several weeks. The unknown reaction within the neurosecretory cells therefore shows the kinetics of a threshold reaction but not of an "all or none" reaction.

We have observed that an intermediate range of low temperatures (6 to 15°C.) is optimal for the physiological transformation of the brain. At temperatures as high as 25° C. or as low as 2½° C. the process is greatly retarded. The fact that 2½° C. is far less effective than 6° C. gives assurance that no single temperature-inhibited reaction (such, for example, as adsorption) will suffice. The minimal requirements appear to be the interaction of two antagonistic reactions with different temperature coefficients, the one favoring and the other opposing the activation of the neurosecretory cells.

Until very recently, the analysis could not be pursued beyond this point. Most fortunately, however, the biochemical and physiological events within the pupal brain of the Cecropia silkworm have just been described in detail by Van der Kloot (1955). In brief, the unchilled diapausing brain is found to be not only endocrinologically incompetent, but also electrically silent—a state which can be accounted for in terms of the apparent absence of cholinesterase from the brain as a whole. The cholinergic substrate of this enzyme (presumed to be acetyl choline) is, at the outset, present in only trace amounts. Exposure to low temperatures greatly accelerates the accumulation of this substrate within the brain. Then, when the chilled pupa is returned to 25° C., the brain shows a prompt, and, presumably, inductive synthesis of cholinesterase. The titer of acetyl choline decreases to an intermediate level, and electrical and endocrine activities immediately reappear. These various changes are peculiar to the brain and are not encountered in any of the other ganglia.

The implications of these extraordinary findings are discussed in detail by Van der Kloot. For present purposes it is of particular interest that the entire brain is shut down at the outset of diapause and reactivated months later under the influence of environmental temperature. What this means in terms of the function of the neurosecretory cells is not clear. Van der Kloot suggests that the entire brain is inactivated to prevent the neurosecretory cells from being driven by nerve impulses at synapses with afferent and internuncial neurons. And one may likewise conjecture that within the neurosecretory cells the synthesis and secretion of hormone continues to require cholinergic mechanisms of the same type as preside over the genesis of nerve impulses and the release of neurohumors.

The primary change induced by chilling therefore appears to be the synthesis and

accumulation within the brain of a cholinergic substrate which appears to be acetyl choline. It will be recalled that molecules such as acetyl choline are notoriously unstable in solution at physiological pH, and one may predict that the accumulation of substrate is opposed by non-enzymatic breakdown. Consequently, it is even possible that the synthesis and breakdown of the cholinergic substrate correspond to the pair of antagonistic, temperature-sensitive reactions whose existence was found necessary to account for the endocrinological activation of the brain.

## Summary

1. The termination of the pupal diapause of the Cecropia silkworm is potentiated by preliminary exposure to low temperature and, especially, to temperatures in the range 6 to 15° C.

2. This effect is mediated by an endocrine mechanism which centers in the brain, and is specifically concerned with restoring the competency of the brain's neurosecretory cells to synthesize and secrete the brain hormone.

3. The time required for the initiation of adult development at 25° C. is thereby reduced from 27 weeks to as little as one day.

4. As soon as the brain has recovered its endocrine function, the catalytic effects of low temperature are at an end; the actual secretion of the hormone and the developmental response that follows are then favored by high temperature. In this manner the low temperatures of winter potentiate the initiation of adult development the following spring.

5. The effects of low temperature on the inactive, diapausing brain are considered in detail. The action is apparently a direct one on temperature-sensitive reactions within the brain itself. The over-all process of activation shows the kinetics of a threshold reaction but not of an all-or-none reaction.

6. These findings are discussed in the light of Van der Kloot's chemical and physiological studies of the brain of Cecropia. Within the substance of the brain the primary change induced by low temperature appears to be the synthesis and accumulation of a cholinergic substrate.

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